

Bacterial Ice Nucleation: A Factor in Frost Injury to Plants¹

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ABSTRACT

Heterogeneous ice nuclei are necessary, and the common epiphytic ice nucleation active (INA) bacteria *Pseudomonas syringae* van Hall and *Erwinia herbicola* (Löhnis) Dye are sufficient to incite frost injury to sensitive plants at -5°C . The ice nucleation activity of the bacteria occurs at the same temperatures at which frost injury to sensitive plants occurs in nature. Bacterial ice nucleation on leaves can be detected at about -2°C , whereas the leaves themselves, i.e. without INA bacteria, contain nuclei active only at much lower temperatures. The temperature at which injury to plants occurs is predictable on the basis of the ice nucleation activity of leaf discs, which in turn depends on the number and ice nucleation activity of their resident bacteria. Bacterial isolates which are able to incite injury to corn at -5°C are always active as ice nuclei at -5°C . INA bacteria incited frost injury to all of the species of sensitive plants tested.

Damage to 'frost-sensitive' plants under natural conditions usually occurs between -2 and -5°C (2, 11, 20). At these temperatures, ice forms from supercooled water in such plants, propagates throughout the plants (inter- and intracellularly), and frost damage occurs. In the absence of sites capable of ice nucleation, the water in plant tissues can supercool; freezing will not occur until the temperature becomes low enough that the most active ice nucleus associated with the plant is able to catalyze crystallization of supercooled water.

Nevertheless, a lack of information on the process of supercooling of sensitive plants (3) has led to differences in opinion as to whether avoidance of frost damage through supercooling is important under natural conditions. Many investigators cite the fact that most sensitive plants do not supercool below -2 to -5°C as a reason that ice avoidance is a minor phenomenon, and occurs only when the temperature falls only slightly below freezing for a very brief period (2, 4, 11). Levitt (11) has pointed out that supercooling has rarely been demonstrated as a mechanism by which sensitive plants avoid frost damage. Mayland and Cary (19) recognized supercooling as a frost protection mechanism. Supercooling may protect blossoms in orchards against spring frost injury (20). Supercooling is probably a factor in avoidance of frost injury by citrus fruits (7, 23). Lucas (15) recognized that lemon tissue does supercool to -4°C and that ice nucleation events on the surface of the tissue determined the extent of supercooling. He suggested 'atmospheric nuclei' as the source of the ice nuclei on

the surface of lemon fruit. Marcellos and Single (18) recognized that wheat plants supercool readily and that external ice nucleation is necessary for frost formation at temperatures above -10°C . They also found that airborne particles or ice crystals were unlikely to account for ice nucleation in these plants.

Ice nucleation activity at about -2°C has been detected in suspensions of some bacteria, including *Pseudomonas syringae* van Hall (16) and *Erwinia herbicola* (Löhnis) Dye (13). INA³ strains of these two bacterial species are widely distributed on leaves of numerous plant species (14). Thus, certain epiphytic bacteria may serve as the ice nuclei that prevent supercooling of plants (12).

Seedling corn plants with leaf surface populations of *P. syringae* were substantially damaged at -3.5 to -4°C , whereas plants lacking this bacterium were not extensively damaged above -8°C (1). The amount of injury at -4°C was directly proportional to the logarithm of the population of an INA *E. herbicola* strain present on corn leaves (13). Thus, at least two common epiphytic bacteria—*P. syringae* and *E. herbicola*—are active as ice nuclei and increase the amount of frost damage to plants at temperatures near -4°C .

In this report, we will address the proposition that the effect of INA bacteria on the apparent frost sensitivity of plants is due to, or associated with, the ice nucleation property of these bacteria. Our case for the sufficiency of bacterial ice nuclei on plants to incite frost injury under natural conditions has appeared elsewhere (12).

MATERIALS AND METHODS

Bacteriological. Most of the authentic cultures of bacteria used in this study were from the culture collection of Arthur Kelman (Department of Plant Pathology, University of Wisconsin-Madison). We have reported the authentication, ice nucleation activities, and ability to incite frost injury to corn by isolate No. 31 of *P. syringae* (1) and isolate No. 26 of *E. herbicola* (13). Field isolates were selected as discrete colonies from dilution platings of leaf washings on nutrient agar containing 2.5% glycerol or on modified Crosse's medium (6). *P. syringae* formed characteristic light blue, domed colonies on modified Crosse's medium; *E. herbicola* isolates active in ice nucleation formed mucoid bluish colonies with yellow patches after 4 d on this medium. All isolates tentatively identified as *P. syringae* had negative arginine dihydrolase and oxidase reactions, and produced a diffusible fluorescent pigment on Kings medium B (5, 9). All putative *E. herbicola* isolates were yellow pigmented and grew in the presence of 5% NaCl or 0.1% triphenyl tetrazolium chloride (10).

Growth of Plant Materials. Plants were grown in vermiculite in growth chambers with an 18-h light period at 30°C and a 6-h dark period at 20°C , and watered daily with a nutrient solution. From 50 to 160 pots (containing 6 seedlings/pot) constituted an experi-

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³ Abbreviation: INA, ice nucleation active.

ment. The following plants were tested: corn (*Zea mays* L. cv W64A × A632); bean (*Phaseolus vulgaris* L. cv Early Galatin); lettuce (*Lactuca sativa* L. cv Buttercrunch); eggplant (*Solanum melongena* L. cv Black Beauty); tomato (*Lycopersicon esculentum* Mill. cv Rutgers); marigold (*Tagetes patula* L.); zinnia (*Zinnia elegans* Jacq. cv Red Riding Hood); pumpkin (*Cucurbita pepo* L. cv Small Sugar); cucumber (*Cucumis sativus* L. cv Straight Eight); barley (*Hordeum vulgare* L.); wheat (*Triticum aestivum* L. cv Minhardi); and sunflower (*Helianthus annuus* L.). All plants were grown to a minimum height of 15 cm before use. Axenic leaf tissue was obtained by growing surface-sterilized seeds (1% NaOCl for 10 min) in deep Petri plates or in 500-ml Erlenmeyer flasks on sterile nutrient solution (which was solidified with 1.0% w/v agar).

Frost Sensitivity of Intact Plants. The measurement of frost sensitivity of intact plants was similar to that previously reported for use with three-leaf-stage corn seedlings (1, 13). Pots were divided randomly into treatment groups (10 pots/treatment) and either sprayed with bacterial suspensions (approximately 10^7 cells/ml) or left untreated. Plants were incubated in a 24°C mist chamber for either 24 h (*P. syringae* treatments and controls) or 48 h (*E. herbicola* treatments and controls). After incubation, plants were allowed to dry briefly; treatments were randomized with respect to position on carts and placed in the freezing chamber at about 0°C. Plants were then cooled slowly (0.1–0.05°C/min) to the minimum temperature required and were held at that temperature for at least 4 min before rewarming to 30°C. Damage was assessed 24 h later and expressed as the fraction of leaves/plant that showed any injury. Twelve thermocouples, distributed about the freezing chamber at leaf height, indicated less than 0.2°C spread about the median air temperature at the time of minimum temperature.

Ice Nucleation Activity Measurement. The single-temperature method of Lindow *et al.* (13) was used for routine determination of ice nucleation activity at –5°C. Bacterial strains were usually tested after 2 to 4 d growth on nutrient agar containing 2.5% glycerol.

Ice nucleation activity spectra of bacterial suspensions were obtained by a droplet-freezing method similar to that of Vali (22), as modified by Lindow *et al.* (12). Absolute temperature determinations were estimated to be accurate to $\pm 0.2^\circ\text{C}$. The cumulative number, $N(T)$, of ice nuclei per unit volume of suspension active at or above a given temperature was calculated by the equation of Vali (22):

$$N(T) = -\ln(f)V^{-1}$$

where f equals the fraction of droplets unfrozen at temperature T , and V the volume of each droplet used. Since the most active ice nucleus in a drop (*i.e.* the agent which catalyzes ice formation at the highest temperature) will determine the temperature at which a given water drop freezes, only the most active ice nucleus in each droplet is detectable. Thus, a series of dilutions was used to detect less active but more numerous ice nuclei in order to obtain the entire ice nucleus spectrum of bacterial suspensions. The ice nucleus concentration in each dilution was normalized to the concentration in the original suspension. Nucleation frequency (NF) is the fraction of cells active at a given temperature (*i.e.* the number of nuclei/cell) and is determined as the number of ice nuclei/ml divided by the cell density (cells/ml).

Ice Nucleation Activity Spectra of Leaf Material. The technique for measuring the ice nucleus spectra of bacterial suspensions was adapted for use with leaf discs. Leaf discs of about 3 mm diameter were cut from random parts of leaves using either a No. 0 cork borer or a paper punch. Individual discs were placed on the cooling surface with a dissecting needle and were either submerged in, or floated on, 30 μl droplets of sterile water. The surface was cooled, and nucleation events in or on leaf discs were sensed by visually noting when the supporting droplet froze. The tempera-

ture at which each of 40 to 70 discs per plant sample catalyzed ice formation was recorded manually. The cumulative number of nuclei per gram of leaf tissue was determined by substituting the mass of each leaf disc (M) for the volume term (V) in the equation noted above. This calculation was independent of the volume used since the water drops themselves did not contain ice nuclei active above -15°C . the calculation assumed that the leaf discs were of equal mass (about 1.5 mg each).

RESULTS AND DISCUSSION

Ice Nucleation Activity of Bacterial Suspensions. The ice nucleation activity of *P. syringae* and *E. herbicola* is illustrated in Figures 1 and 2. In Figure 1, the cumulative fraction of droplets frozen in each of a series of five 10-fold dilutions is plotted as a function of decreasing temperature. The ice nucleation spectra from these data are shown in Figure 2. Ice nucleation was detected at -2.3°C in the *P. syringae* (isolate No. 31) suspension (Fig. 1A). As the temperature decreased from -2.3 to -4°C , the nucleation frequency increased from $<10^{-7}$ to about 10^{-2} . Little additional increase in nucleation frequency occurred between -4 and -9°C . Although the overall shape of the ice nucleation spectrum in Figure 2A is typical of those determined for *P. syringae* isolates grown on this medium, the nucleation frequencies of different

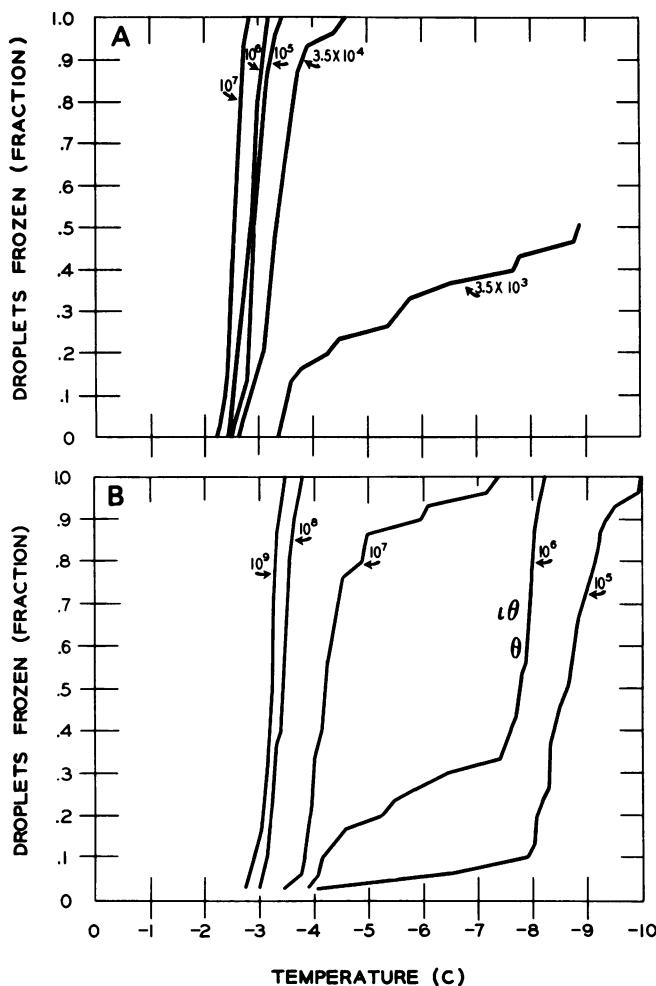


FIG. 1. Freezing spectra of cell suspensions of (A) *P. syringae* isolate No. 31 and (B) *E. herbicola* isolate No. 26. Cells were grown on nutrient agar containing 2.5% glycerol for 2 d at 20°C and suspended in sterile water to yield suspensions of 3.5×10^7 *P. syringae* cells/ml and 1.6×10^9 *E. herbicola* cells/ml. The curves represent the freezing of 30 droplets from each of five 10-fold dilutions.

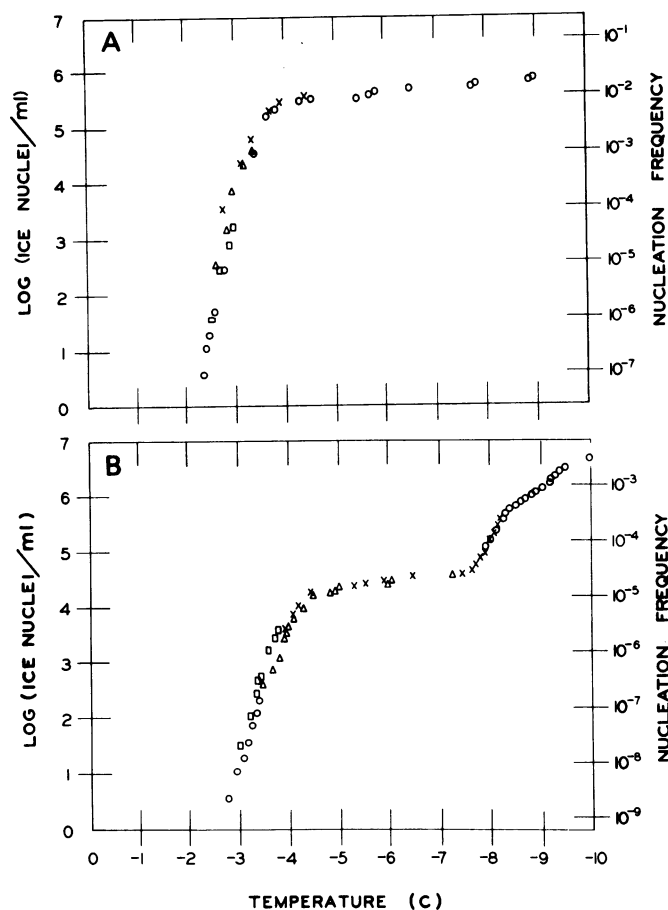


FIG. 2. Cumulative ice nucleus concentration and the fraction of cells active in ice nucleation at or above a given temperature for (A) *P. syringae* and (B) *E. herbicola* determined from data in Figure 1. Each symbol represents determinations from one of the five different dilutions.

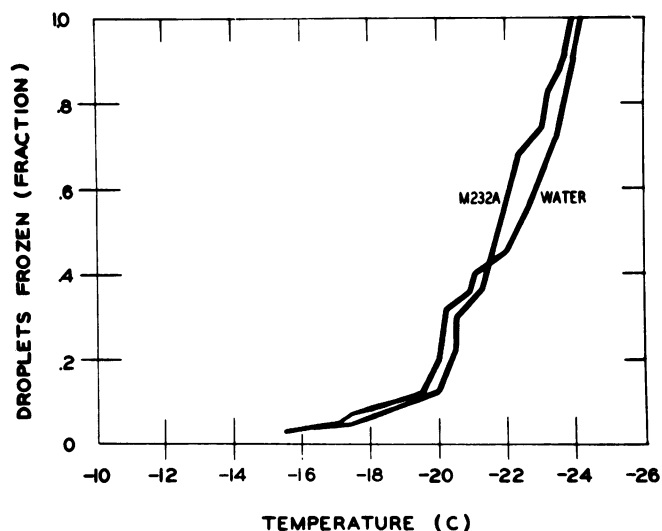


FIG. 3. Freezing spectra associated with distilled H_2O or with a suspension of 1×10^7 cells/ml of the non-ice nucleation active bacterium *E. herbicola* isolate M232A. The cumulative fraction of 50 μ l-droplets which froze is plotted as a function of decreasing temperature.

isolates at any given temperature can be very different.

Ice nucleation was first detected in the suspensions of *E. herbicola* isolate No. 26 at $-2.6^\circ C$ (Fig. 1B). In suspensions of *E. herbicola*, there were two temperature ranges in which there was

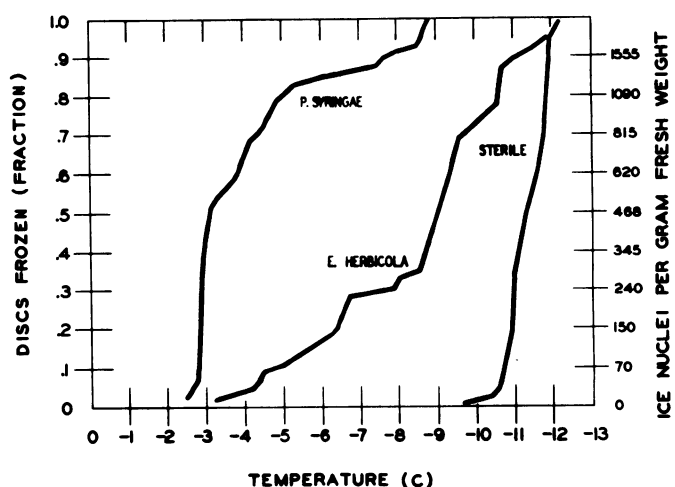


FIG. 4. Ice nucleation activity of corn leaf discs from plants with and without leaf surface populations of *P. syringae* and *E. herbicola*. Corn seedlings grown in sterile Hoagland nutrient solution were sprayed with suspensions of 2×10^8 cells/ml of *P. syringae* isolate No. 31 or 3×10^7 cells/ml of *E. herbicola* isolate No. 26 in phosphate buffer or with buffer alone and incubated in a mist chamber. The cumulative fraction of discs which froze (left axis) or ice nucleus content (right axis) was plotted as a function of decreasing temperature.

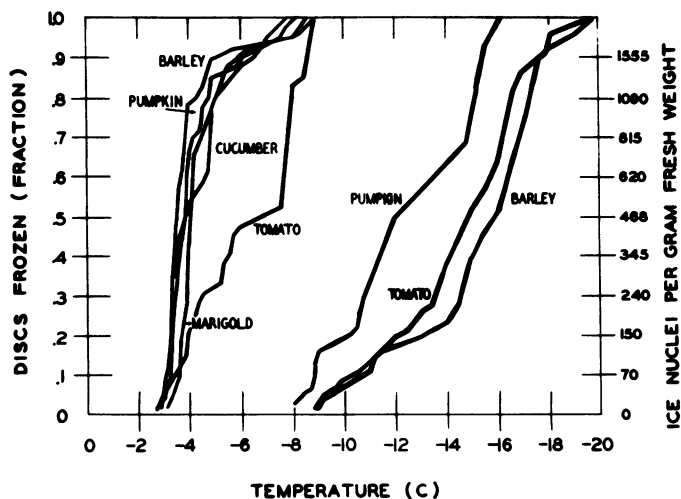


FIG. 5. Ice nucleation activity of leaf discs of pumpkin, cucumber, marigold, barley, and tomato seedlings sprayed with about 2×10^8 cells/ml *P. syringae* isolate No. 31 in phosphate buffer and incubated in a mist chamber for 24 h before assay (left group). These were compared with discs from pumpkin, barley, and tomato seedlings grown axenically on sterile Hoagland solution (right group). The cumulative fraction of discs that froze (left axis) and the ice nucleus concentration (right axis) are plotted as functions of decreasing temperature. Since values of nuclei per g vary with weights of the leaf discs and weights of 3 mm discs taken from different plants differ slightly, the absolute values of nuclei per g shown may be slightly incorrect; relative values are correct within each curve.

a marked increase in nucleation activity with decreasing temperature. In the first (region I), between -2.6 and $-4^\circ C$, the nucleation frequency increased from about 10^{-8} to about 10^{-5} (Fig. 2B). Little increase in nucleation frequency occurred between -4.5 and $-7.5^\circ C$. In the second (region II), from -7.5 to $-9.5^\circ C$, the nucleation frequency increased from about 10^{-5} to $>10^{-3}$. Below $-10^\circ C$, no additional increase in ice nucleation by either bacterium was detected. Thus, we have restricted our study of bacterial ice nucleation to temperatures between 0 and $-10^\circ C$. Within this temperature range, only a very small fraction of the cells in each

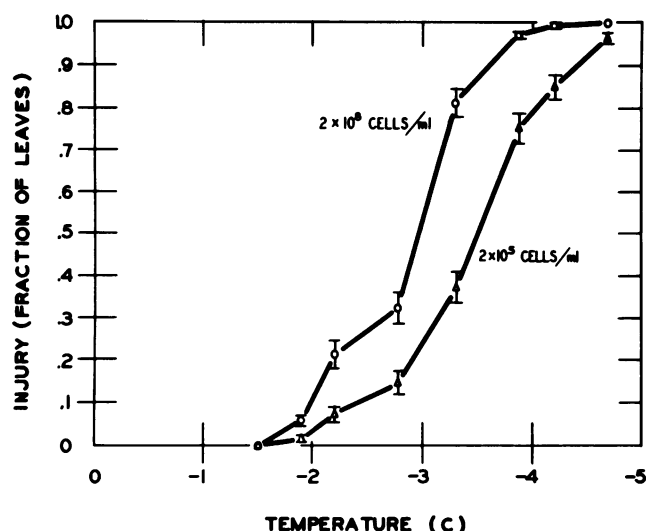


FIG. 6. Frost damage to corn seedlings as a function of temperature at two *E. herbicola* population levels on leaves. Plants were sprayed with 2×10^8 (○) or 2×10^5 (△) CFU/ml suspensions of *E. herbicola* in phosphate buffer, held in a mist chamber for 48 h, and subjected to the temperatures shown. Vertical bars represent SE.

culture were active—1/several hundred for *E. herbicola* and 1/50 in the *P. syringae* suspension. This appears to be typical of the INA bacteria examined to date (8).

Erwinia herbicola No. 26 produced fewer ice nuclei per cell than did *P. syringae* No. 31, particularly at temperatures above -5°C (compare Figures 2A and 2B). In addition, ice nucleation in the *E. herbicola* suspensions was first detected at a slightly lower temperature than in the *P. syringae* suspension. Thus, on a per-cell basis, *E. herbicola* will be less effective than *P. syringae* in preventing supercooling at temperatures above -5°C . The cells active in the high temperature range (region I) are the ones likely to be involved in frost damage since frost injury to sensitive plants usually occurs at temperatures above -4 to -5°C . Both *P. syringae* and *E. herbicola* are sources of ice nuclei active at temperatures warm enough to be associated with frost injury. All of the 'non-ice-nucleating' bacterial isolates for which the ice nucleation spectrum has been determined had spectra which were indistinguishable from that of water under our test conditions. This is illustrated by the nearly identical spectra of a very dense suspension of the inactive *E. herbicola* isolate M232A and of water shown in Figure 3. Droplets of water or of the M232A suspensions did not begin to freeze until the temperature was below -15°C . Substantial ice nucleation activity was not detected until the temperature had dropped to -20°C . Nucleation at or below this temperature was due either to the presence of impurities in the water, or more likely, from ice nucleation events occurring on the cooling surface.

Ice Nucleation Activity of Plant Material. The ice nucleation activity of leaf tissue was measured by observations of the temperature at which water droplets in which discs of leaf tissue were suspended froze, and was quantitated by analysis of freezing temperatures of a collection of such discs. There is evidence (15, 18), at least in frost-sensitive plants, that ice crystals will propagate from plant tissue into water in contact with the tissue. We have observed that corn leaf discs retrieved from droplets that had frozen invariably exhibited the water-soaked appearance characteristic of frost damage, while discs from droplets which did not freeze at temperatures of -10°C or lower remained normal in appearance.

Two major assumptions were made in derivation of the equation (22) used for calculation of a cumulative ice nucleus concentration: (a) that time dependence is only of secondary importance in the nucleation process and (b) that each nucleus has a specific nuclea-

Table 1. Comparison of INA Bacteria and non-INA Bacteria as Incitants of Frost Damage to Seedling Corn at -5°C

Bacterial Species ^a	Bacterial isolates as incitants of frost injury	
	Injury ^b	No injury ^c
	<i>No. of isolates</i>	
Isolates active as nuclei at -5°C		
<i>Pseudomonas syringae</i>	81	2
<i>P. syringae</i> -like field isolates ^d	28	0
<i>P. syringae</i> pv. <i>coronafaciens</i>	6	0
<i>P. syringae</i> pv. <i>pisi</i>	4	0
<i>P. syringae</i> pv. <i>lachrymans</i>	5	0
<i>P. fluorescens</i>	1	0
<i>Erwinia herbicola</i>	2	0
<i>E. herbicola</i> -like field isolates ^e	41	5
Total INA isolates tested	168	7

Isolates inactive as ice nuclei at -5°C

<i>Pseudomonas syringae</i>	0	4
<i>P. syringae</i> pv. <i>coronafaciens</i>	0	1
<i>P. syringae</i> pv. <i>glycinea</i>	0	1
<i>P. syringae</i> pv. <i>tabaci</i>	0	3
<i>P. syringae</i> pv. <i>phaseolicola</i>	0	2
<i>P. marginalis</i>	0	1
<i>P. fluorescens</i>	0	2
<i>P. aeruginosa</i>	0	1
<i>P. solanacearum</i>	0	1
<i>Erwinia stewartii</i>	0	4
<i>E. carotovora</i> var. <i>carotovora</i>	0	2
<i>E. chrysanthemi</i>	0	2
<i>E. herbicola</i>	0	4
<i>Escherichia coli</i>	0	1
<i>Xanthomonas campestris</i>	0	2
<i>X. axonopodia</i>	0	1
<i>Corynebacterium nebraskensis</i>	0	4
Miscellaneous other isolates	0	52
Total non-INA isolates tested	0	88

^a Most identified isolates supplied by A. Kelman.

^b Damage significantly greater than in controls.

^c Damage not significantly greater than in controls.

^d *P. syringae*-like field isolates all had the appropriate colony and physiological characteristics (5), including levan formation on modified Crosse's medium (6), were fluorescent on Kings medium B (9), and were oxidase negative.

^e *E. herbicola*-like field isolates from corn and soybean during 1975. All were yellow pigmented, had colony morphologies resembling those of known INA *E. herbicola* isolates, and including characteristic mucoid growth on modified Crosse's medium.

tion temperature. Ice nucleation events on the surface of leaf discs should follow these assumptions as well as nuclei in suspensions. It might be argued, however, that a delay may occur between a nucleation event within a leaf disc and freezing of the water droplet surrounding it. We have found that ice will propagate at a minimum velocity of about 5 cm/min in corn leaf tissue supercooled to -5°C , and that greater velocities might actually be more probable. Single (21) has reported ice propagation within plant tissue of over 100 cm/min at -5°C . At 5 cm/min, less than 2 s would be required for ice nucleated within the 3 mm discs to propagate to the disc-droplet interface and initiate freezing of the droplet. Since cooling rates of less than 0.3 $^\circ\text{C}/\text{min}$ were used in this study, errors due to possible delay of disc freezing are probably minimal.

This method does not distinguish between nucleation events which may occur on the surface of the leaf discs or within the leaf

disc tissue. However, the propagation of ice crystals within our system and the likelihood that some water will be present on leaves under conditions under which natural frost would be likely to occur apparently make this distinction unnecessary. Thus, the leaf disc systems seems to be one which will accurately measure the ice nucleation activity of leaf tissue (including associated INA bacteria) under conditions where ice nucleation of plant material would be of concern in nature.

Figure 4 illustrates the ice nucleation activity spectra associated with corn leaf discs. Corn tissue grown under sterile conditions did not contain detectable nucleation activity above approximately -10°C and ice nucleation occurred over the narrow temperature range of about -10.5 to -12°C . Corn tissue grown in a growth chamber under conditions which avoided establishment of either *P. syringae* or *E. herbicola*, but were in no way axenic, apparently did not contain ice nuclei active above about -8°C , since frost injury to the plants occurred at about that temperature (1). The ice nucleation threshold of corn tissue which had been sprayed with suspensions of approximately 10^8 cells/ml of *P. syringae* was about -2.5°C and of those sprayed with *E. herbicola* was approximately -3°C . At -5°C , corn leaf material with *P. syringae* and *E. herbicola* populations contained about 1,000 nuclei/g and 85 nuclei/g, respectively. Because these two bacterial strains usually achieve similar populations on the leaf surface, the higher ice nucleus concentration on *P. syringae*-sprayed leaves than on *E. herbicola*-sprayed leaves is consistent with the higher nucleation activity of *P. syringae* at -5°C .

The ice nucleation activity of some other frost-sensitive plants is illustrated in Figure 5. Leaf discs from tomato plants which had been sprayed with suspensions of approximately 10^8 cells/ml of *P. syringae* contained active nuclei in the temperature range of -2.5 to -4.0°C (detectable threshold, -2.5°C). Leaf discs of sterile tomato leaves had an ice nucleation threshold of -9°C . Leaf discs of *P. syringae*-sprayed marigold, pumpkin, cucumber, and barley also contained active nuclei, with typical thresholds of about -2.8°C . Leaf discs of pumpkin and barley free of INA bacteria did not contain nuclei active at temperatures above -8°C . A similar lack of ice nucleation activity above -8°C has been found in leaf discs from wheat, sunflower, bean, and eggplant. Discs of all these plants had ice nucleation thresholds above -3°C after application of INA bacteria.

Thus, we were unable to detect ice nucleation activity at temperatures warm enough to be associated with 'natural' frost in leaves of any of the plant species when bacterial ice nuclei were not present. Thus, we conclude that leaves do not contain intrinsic ice nuclei measurable by this technique that are active at temperatures above about -8°C . Plants lacking ice nuclei active at -5°C should not freeze at or above that temperature. However, when INA bacteria were present on the leaves, ice nucleation was detectable at the same temperatures at which suspensions of the bacteria are active (compare Figs. 1, 2, and 4). In plants bearing such nuclei, ice would form at the temperatures at which 'natural' frost injury occurs.

Freezing Temperature of Plant Material and Its Dependence on INA Bacteria. The extent of frost damage to corn seedlings at a given temperature is a function of the INA bacterial populations on the leaves at the time of freezing. In an earlier report (12), we have shown that plants with leaf surface populations of *P. syringae* sustained some damage at temperatures only slightly below -2°C ; damage increased with decreasing temperature until nearly all leaves were injured at about -3 on plants sprayed with suspensions of about 10^7 cells/ml or at -4°C on plants with about 10^6 cells/ml. Similarly, plants with *E. herbicola* on their leaf surface sustained some frost injury between -2 and -3°C ; damage increased with decreasing temperature and was complete by -4°C on plants sprayed with dense (2×10^8 cells/ml) or by -5°C on plants sprayed with dilute (2×10^6 cells/ml) cell suspensions (Fig. 6).

Thus, at comparable cell densities, extensive damage occurred about 1°C colder to plants treated with *E. herbicola* as compared with plants treated with *P. syringae*. This is consistent with the slightly lower ice nucleation activity of *E. herbicola* as compared to *P. syringae* between -2 and -5°C . By comparison, untreated corn seedlings grown in our growth chambers were not damaged above -7°C and damage was not complete until about -9°C (1). The total bacterial populations present on the leaves of these untreated plants were in the range of 10^3 to 10^4 /g fresh weight and INA bacteria were rarely found on the leaves of these control plants. Thus, the amount of damage at any given temperature between -2 and -8°C is substantially influenced by the number of INA bacteria of either species present on the leaves at the time of freezing. The threshold temperature for frost damage is very close to the threshold for ice nucleation by the bacteria in suspensions (compare Figs. 2B and 6).

Increased Frost Sensitivity of *P. syringae* and *E. herbicola* Sprayed Plants. The association between the presence of *P. syringae* or *E. herbicola* and frost injury to corn at -5°C has been established (1, 12, 13). However, when suspensions of either *P. syringae* or *E. herbicola* of approximately 10^7 cells/ml were sprayed on other plants considered to be frost-sensitive 48 h before freezing at -4 to -5°C , an increase in frost damage to these plants, compared to plants without the bacteria, was observed. These different plants included tobacco, bean, lettuce, marigold, eggplant, sunflower, tomato, zinnia, pumpkin, and cucumber. In all cases, almost no damage to plants sprayed with buffer alone was observed after freezing at -5°C , whereas plants sprayed with *P. syringae* or *E. herbicola* were almost completely killed at this temperature. Since we have not yet found a normally frost-sensitive plant that is not so affected, the range of activity of these bacterial ice nuclei is clearly not restricted to a narrow group of plants, i.e. the ability of both *P. syringae* and *E. herbicola* to increase the damage to plants at temperatures between -2 and -5°C is probably quite general in nature. The control plants in our growth room experiments are 'unnatural' because they do not have an epiphytic population of INA bacteria. Thus, they are not injured at temperatures (-2 to -5°C) at which 'natural' plants in the field, with the normal population of epiphytic INA bacteria, would sustain freezing damage (14).

INA Bacterial Isolates and Increased Frost Damage. If the hypothesis that the ice nucleation activity of bacteria is involved in the process of inciting frost damage to plants is correct, then only bacteria with ice nucleation activity should incite frost injury. Therefore, the ability of 264 strains of bacteria to incite frost injury to corn seedlings at -5°C was compared to their ice nucleation activity (Table I). Several epiphytic bacteria were selected from dilution plates of leaf washings of corn, beans, soybean, and cherry during the 1975 and 1976 growing seasons. The collection of *P. syringae* strains identified by others had been isolated, frequently as pathogens, from plants as diverse as sour cherry (*Prunus cerasus* L.), peach (*Prunus persica* (L.) Batsch), apricot (*Prunus americana* Marsh), bell pepper (*Capsicum annuum* L.), bean (*Phaseolus vulgaris* L.), onion (*Allium cepa* L.), wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), lilac (*Syringa vulgaris* L.), pear (*Pyrus communis* L.), and hairy vetch (*Vicia villosa* Roth). Nearly all *P. syringae* isolates were active in ice nucleation (83 of 87). Some, but not all, phytopathogenic pseudomonads now classified as *P. syringae* (5) were found to be active in ice nucleation at -5°C . Although the numbers of isolates of these related pseudomonads were small, only isolates of *P. syringae* pv. *coronafaciens*, *P. syringae* pv. *pisi*, and *P. syringae* pv. *lachrymans* were active, whereas other *P. syringae* pathotypes were not, i.e. *P. syringae* pv. *glycinea*, *P. syringae* pv. *tabaci*, and *P. syringae* pv. *phaseolicola*. Except for one *P. fluorescens* isolate obtained from L. R. Maki (17), only bacteria identifiable as *P. syringae* (according to the usage of Doudoroff and Palleroni [5]) or *E. herbicola* were

found to be active in ice nucleation at -5°C . Of the 83 *P. syringae* isolates active in ice nucleation, 81 incited frost damage to corn. Similarly, 43 of 48 isolates of *E. herbicola* active in ice nucleation also incited frost damage to corn. Thus, 168 of 175 INA bacterial isolates incited frost damage to corn while none of 88 non-INA bacterial isolates incited frost damage. The INA bacterial isolates which did not incite frost damage to corn may not have been adapted for an epiphytic existence on corn as all were isolated from plants other than corn.

Thus, a strong correlation exists between ice nucleation activity of bacteria and ability to incite frost damage; ice nucleation activity appears to be a necessary but not sufficient attribute enabling bacteria to incite frost injury. As noted above, frost-sensitive plants are damaged upon formation of ice within the tissue; they must avoid ice formation to avoid frost damage. Substantial supercooling is not observed in these plants in nature; they are damaged by ice formation at -2 to -5°C . Plants can supercool below this point only if heterogeneous ice nuclei (INA bacteria) are not present.

The data presented above indicate that the following five points can be added to those arguments that implicate INA bacteria as incitants of frost injury to sensitive plants. First, the ice nucleation activity of the bacteria occurs at temperatures associated with frost injury in nature. Second, bacterial ice nucleation can be detected on plant leaves at about -2°C ; leaves themselves do not appear to be active ice nuclei until much lower temperatures (-9 to -11°C) are reached. Third, the temperature at which frost injury to plants occurs is predictable on the basis of the ice nucleation activity of leaf discs. The ice nucleation activity of leaf discs is predictable on the basis of the number and ice nucleation activity of the bacteria they harbor. Fourth, the bacterial strains able to incite injury at -5°C are always active as ice nuclei at -5°C ; nearly all bacteria that are active ice nuclei at -5°C incite injury at that temperature, at least to corn. Fifth, the ability of INA bacteria to incite frost injury to 'frost-sensitive' plants is quite general; INA bacteria incited frost injury to all of the species of sensitive plants included in our experiments. Thus, heterogeneous ice nuclei are necessary to produce frost injury above -5°C , and epiphytic INA bacteria are sufficient to fulfill this role.

These findings, taken together with the ubiquity of INA bacteria on plants in nature (14) and the fact that most, if not all, ice nuclei active at -2 to -5°C present on leaves are associated with INA bacteria (12), are strong evidence that INA bacteria are incitants of natural frost injury to sensitive plants at -2 to -5°C .

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